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Preliminary communication

Simple isolation of α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc*p*(G_{M3}o.s.) from bovine colostrum through lactonization

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Sialyloligosaccharides of glycoproteins and glycolipids have been associated with a variety of biological processes[1]. Among these, it was recognized that the (2→3) or (2→6) sialic acid linkages with the neighboring galactosyl residues play critical roles in the receptor specificities of different human influenza virus isolates[2]. It therefore appeared of interest to have readily available access to sufficient quantities of simple sialyloligosaccharides of defined linkages for binding and inhibition studies.

The literature is well documented in regard to procedures for the isolation of sialyloligosaccharides (o.s.) from bovine colostrum[3,4] or human milk[5], and for their chemical[6] or enzymic synthesis[7,8]. However, none of these procedures are rapid and simple enough for the easy preparation of the α-D-Neu*p*5Ac-(2→3)-β-D-Gal sequence present in the G_{M3}o.s. (**1a**). We herein report an isolation protocol which is based on the realization that, among the monosialyloligosaccharides present in bovine colostrum[3,4], G_{M3}o.s. is the only major species possessing the appropriate linkage for lactonization. This process transforms G_{M3}o.s. into a neutral species that cannot bind to anion-exchange resins, and so it leads to a simplified purification scheme[3–5]. The corresponding α-D-Neu*p*5Gc-(2→3)-β-D-Gal*p*-(1→4)-D-Glc*p* is known[3] to be present in small proportions in colostrum (<19 mg/L; Neu*p*5Ac: Neu*p*5Gc, 17:1 w/w), and because it should also lactonize by the same procedure, care was taken to ascertain its absence in the purified G_{M3}o.s. We also used ^{1}H-n.m.r. spectroscopy to show that the internal esterification occurs at O-2 of the D-galactosyl residue. As this phenomenon also occurs in the integral G_{M3} ganglioside[9,10], it appears that this mode of lactonization is a particular behavior of the sialyl-(2→3)-galactose sequence. Thus, the actual methodology describes a useful application of this concept and uses it to isolate the G_{M3}o.s (**1a**) present in bovine colostrum.

The isolation procedure is depicted in Scheme 1. Crude bovine colostrum (1

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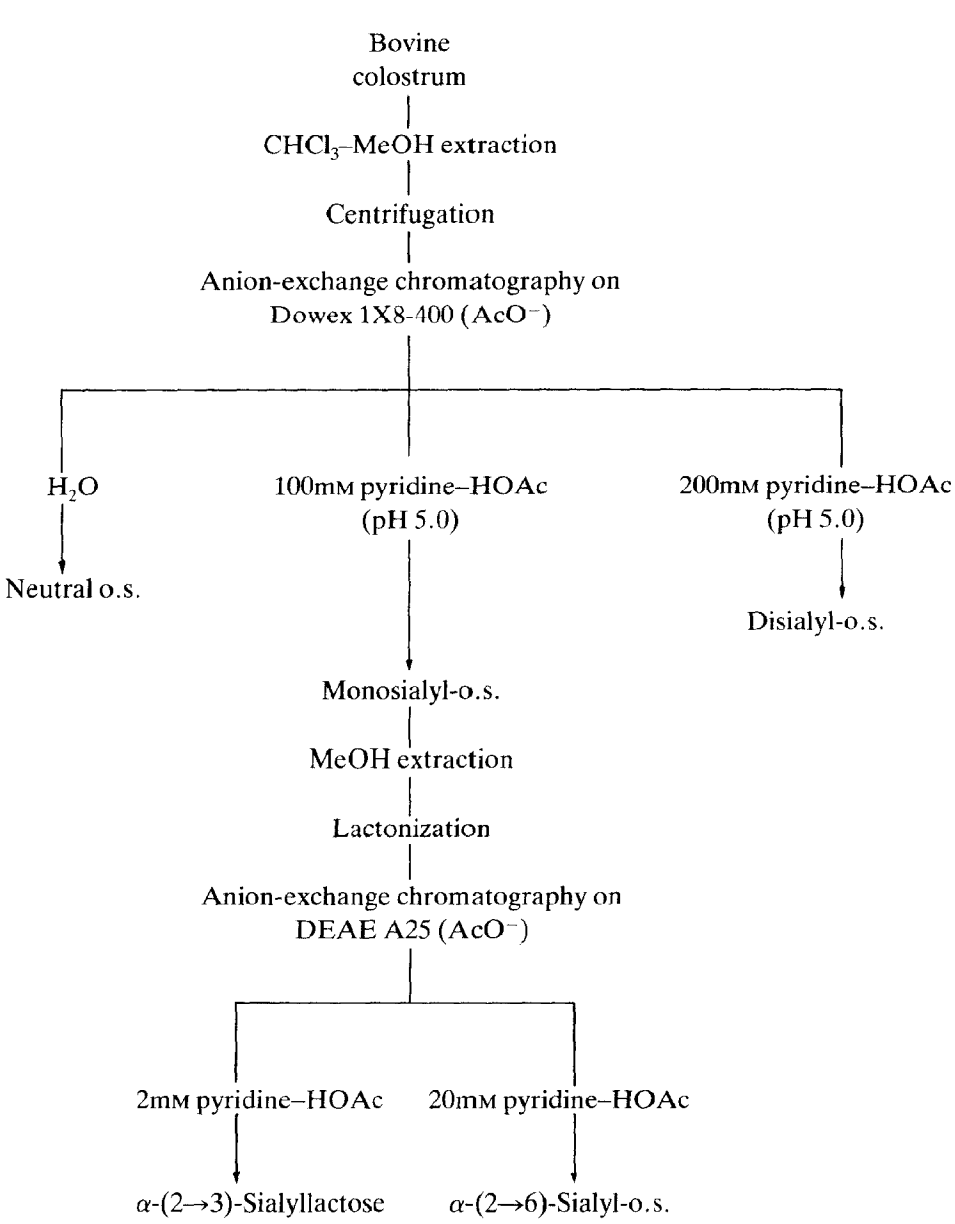
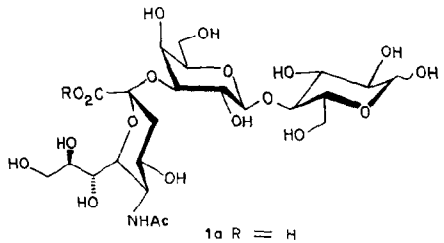
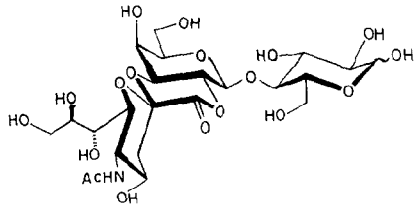


Scheme 1.

L) was shaken with 3:1 (v/v) chloroform–methanol (1 L) until the mixture was homogeneous. Centrifugation at 5000 r.p.m. for 15 min at 4° resulted in three phases; a top aqueous phase, a solid middle plug (denatured proteins), and a bottom organic phase. The aqueous phase was then collected and concentrated to 500 mL in a rotary evaporator. The solution was loaded onto a column (3.0 × 60



1a R = H
1b R = Me
1c R = NH_2
1d R = NHMe



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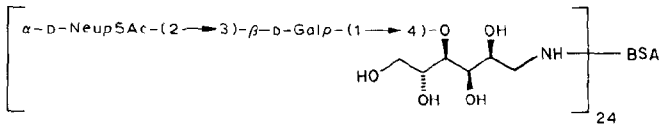

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cm) of Dowex 1X8-400 (AcO^-) ion-exchange resin. The neutral oligosaccharide fraction was eluted with water, and the crude monosialyloligosaccharide fraction with 100mM pyridinium acetate buffer, pH 5.0. The disialyloligosaccharide fraction was eluted, if needed, with a 200-mM concentration of the same buffer. The individual fractions were then lyophilized. At this stage, they could be processed according to published procedures, to afford the well separated oligosaccharides[3-5].

The crude powder containing the monosialyloligosaccharides (0.8–1 g) was extracted two or three times with methanol, to remove residual ninhydrin-positive material. The methanol extracts were evaporated under vacuum. The lactonization of the G_{M3}o.s. (**1a**) was allowed to proceed in glacial acetic acid for 3 d at room temperature, and the mixture was lyophilized. The extent of lactonization could be easily monitored by t.l.c. in 8:2 (v/v) 1-propanol–water. The following compounds and their respective R_F values were observed as follows: **1a**, R_F 0.21; **2** (lactone), R_F 0.34, and α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-D-Glc*p* (II[6] Neu*p*5Ac-Lac), R_F 0.18. The corresponding α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-D-Glc*p*NAc, although present, could not be well differentiated from **1a** in this system. The lyophilized reaction mixture was dissolved in 2mM pyridinium acetate buffer, and loaded onto a column (2.0 × 20 cm) of Sephadex-DEAE A-25 (acetate form). The neutral lactone **2** was not retained, while the remaining crude monosialyl-α-(2→6)-oligosaccharides were eluted with a 20-mM concentration of the same buffer. The yield of pure **2** obtained after lyophilization ranged from 180–230 mg per L of colostrum. Compound **2** had $[\alpha]_D$ + 10.5° (*c* 1.0, 0.1M pyridinium acetate), and showed a characteristic band at 1750 cm^{-1} in the i.r. spectrum (KBr). The lactone **2** was hydrolyzed to **1a** with 0.1M NaOH. The known G_{M3}o.s. (**1a**) thus obtained had $[\alpha]_D$ +20.6° (*c* 0.7, 0.1M pyridinium acetate); negative f.a.b.-m.s.: *m/z* 632 $[(M - 1)^-$ calc. for $C_{23}H_{39}NO_{19}]$. The ^1H-[7,11] and ^{13}C-n.m.r.[12] spectra in D_2O on a Varian XL-300 n.m.r. spectrometer clearly proved the identity and the purity of **1a**

obtained by the foregoing procedure. Examination of the ^{1}H- and ^{13}C-n.m.r. spectra, h.p.l.c. and h.p.t.l.c. of **1a** failed to reveal a detectable amount of the *N*-glycolyl sialic acid analogue.

To determine which hydroxyl group of the D-galactosyl residue in **1a** was involved in the lactone formation, we undertook an exhaustive ^{1}H- and ^{13}C-n.m.r.-spectroscopic analysis of both compounds **1a** and **2** in either D_2O or 49:1 (v/v) Me_2SO-d_6–D_2O. The latter solvent system was chosen as it had been found[10] to give better spreadings of the n.m.r. signals than D_2O. The assignments of most of the ^{1}H and ^{13}C resonances were made by using both homo- and hetero-nuclear shift correlated 2-D n.m.r. (COSY) experiments. Upon comparison of the ^{1}H-n.m.r. spectrum of **1a** with that of **2**, it was clear that O-2 of the D-galactosyl residue was lactonized. The H-2 resonance was shifted from 3.34 p.p.m. for **1a** to 4.70 p.p.m. for **2**. This corresponds to a proton lactonization shift of 1.36 p.p.m., similar to the one observed[10] for G_{M3}. The downfield shifts (0.11–0.35 p.p.m.) also measured for the other protons of the D-galactosyl residue clearly confirmed this. Difference n.O.e. and 2-D n.O.e. (NOESY) experiments on **1a** were done to determine its solution conformation in D_2O. Measurable n.O.e. values between H-3*ax* but not with H-3*eq* of the sialic acid were obtained with H-3 of the galactose. This suggests that the conformation of the sialic acid relative to that of the galactose in **1a** is similar to that found in G_{M1}, G_{M3}[13], and G_{M4} gangliosides[14] and its parent α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p* disaccharide[14]. The conformation of the lactone ring in **2** is depicted as shown for the sake of clarity only. In fact, due to the absence of observable n.O.e.s between H-3 of Gal and either of the H-3s of the Neu5Ac, it is likely that the lactone ring exists in a distorted conformation.

The potential of the foregoing procedure in obtaining sufficient quantities of **1a** for chemical transformations was illustrated by the synthesis of derivatives **1b**–**1d**, as well as of the antigenic neoglycoconjugate **3**. Compounds **1b**–**1d** were prepared by the appropriate, nucleophilic ring-opening of the lactone **2** with NaOMe, NH_3, and H_2NMe, respectively†. The conjugation of **1a** to bovine serum albumin (BSA) was accomplished by direct reductive amination under improved borate buffer conditions, using sodium cyanoborohydride[15].

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†Spectroscopic data (f.a.b.=m.s. and ^{1}H-n.m.r.) agreed with the structures proposed.

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